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A comparative study on spore germination of *Diplazum esculentum* (Retz.) Sw. "Pako Fern" using different potting media

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ABSTRACT

Diplazium esculentum is an economically important species of fern under the family of Athyriaceae, commonly known as the vegetable fern or Pako (Semwal *et al*., 2021). Studies showed that *D. esculentum* is an excellent source of food and phytochemical compounds. Many methods and techniques have been utilized in the propagation of ferns. However, no prior studies have delved into the propagation of spores in soil media for *D. esculentum*, thus this study investigated the spore development of *Diplazium esculentum* using various potting media. The objectives of this study were to compare and determine which potting media yields the highest growth percentage of *D. esculentum* and to identify the duration of germination of *D. esculentum* spores until the gametophyte stage. Five substrates were utilized for the experiment, namely, control group with regular potting mix, carbonized rice hull, coco peat, peat moss, and dried tree fern trunks. Evident growth resulted in the potting media containing carbonized rice hull with 12.21% growth and coco peat with 3.91% growth. No gametophyte growth occurred in media containing dried tree fern trunks and peat moss. Results also showed the importance of soil pH ranging from 5–6 to the spore germination of *D. esculentum*. Analysis of variance showed that there was a significant difference between the gametophyte growth.

Keywords: Spore germination, *Diplazium esculentum*, potting media.

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INTRODUCTION

Ferns have been part of our ecosystem for billions of years and have survived extreme environmental conditions (Wallace *et al*., 1996). However, in terms of economic value, ferns are, unfortunately often ignored (Mannan *et al*., 2008). Recent studies have shown the various economic uses of ferns as an important food source, medicine, insecticide, and ornamental plant (Mannan *et al*., 2008; Badola, 2010; Junejo *et al*., 2015; Koniyo *et al*., 2021).

Diplazium esculentum, a member of the Athyriaceae family is widely known as the vegetable fern or Pako and holds economic importance (Semwal *et al*., 2021). Studies showed that *D. esculentum* is an excellent source of food nutrients and phytochemicals (Badola, 2010; Junejo *et al*., 2015; Koniyo *et al*., 2021). Moreover, it has been found that *D. esculentum* has the potential for the development of pharmaceutical products because of its medicinal properties (Rout *et al*., 2009; Roy & Chaudhuri, 2020; and Koniyo *et al*., 2021). In fact, due to its nutrient-dense nature, it has the potential to help overcome malnutrition (Sharma and Kumar, 2021). Thus, it is important to propagate *D. esculentum* for its economic significance.

Numerous methods and techniques were utilized in the propagation of ferns. Micropropagation can be applied to produce species of fern that are hard to propagate conventionally for the benefit of the ornamental industry (Khan *et al*., 2008), and with the influx of various media, many botanists are using tissue culture (Morel and Wetmore, 1951; Padhya *et al*., 1982; Hicks and Aderkas, 1986; Makowski *et al*., 2016). However, tissue culture is costly and requires tedious work because all variables that affect the propagation in using this technique are controlled and can only be used for a certain number of species (Debergh and Maene, 1981).

Apart from tissue culture, many other techniques have been used, such as slide culture (Davies *et al*., 1948; Fernandez *et al*., 1999) and rhizome propagation (Lewandowski, 1998; Nivot *et al*., 2008; Boersma, 2014;). However, no studies explored spore propagation using different media for *D. esculentum*. Therefore, this study aims to investigate the development of *Diplazium esculentum* spores using different potting media.

MATERIALS AND METHODS

Identification of Samples

The study was conducted to investigate the growth of *Diplazium esculentum* with the use of different potting media. Thus, the ferns used in this study must be the correct species. The pako fern was observed as freely growing beside a small water source (water canal). The collected ferns have the following characteristics: sori are linear along the basal of ultimate costules (Figure 1), fronds are pinnatifid, and rhizomes are erect and creeping. The mature spores are found on the abaxial side of the sporophyll leaf and are indicated by the black or brown-colored angium (Figure 1). The fronds were removed using pruning shears. The

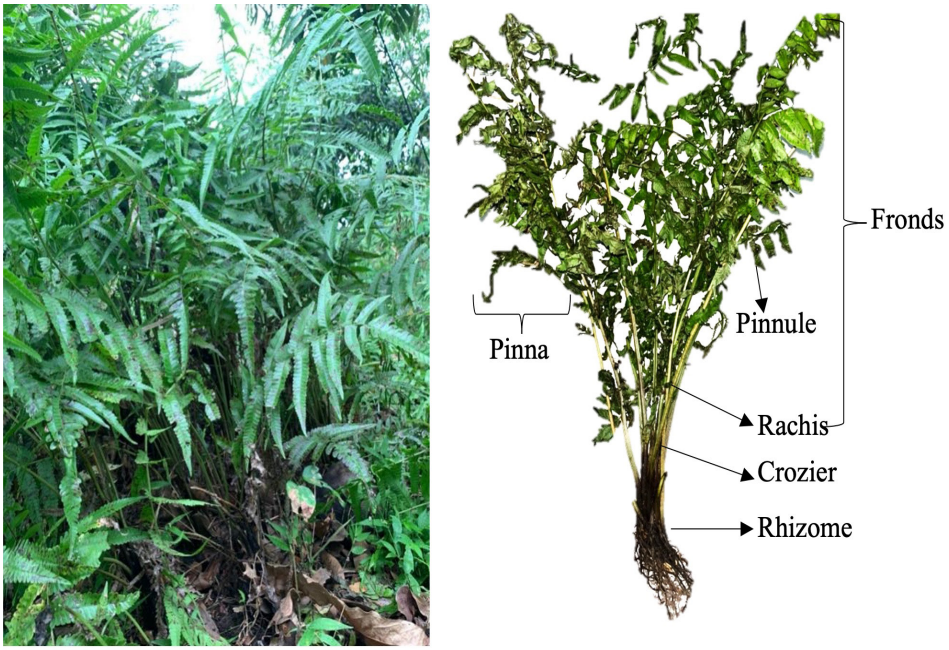

Figure 1. Habit of *Diplazium esculentum* (left) Labeled Structure of *Diplazium esculentum* (right)

samples were identified using the book of Amoroso *et al*. (2016).

Study Site

The samples were collected at the Municipality of Valencia, Negros Oriental (coordinates:9°16'59"N, 123°11'27"E). Valencia is a municipality in the province of Negros Oriental, located 9 kilometers or 5.6 miles west and uphill of the provincial capital Dumaguete City. Paco ferns were observed as freely growing beside a small water source (water canal) near the Forest Camp and alongside the downstream of the "Kawa" river. A random collection of mature *D. esculentum* spores and fertile fronds was conducted in both locations (Fig. 2).

Collection of Samples

Fronds of *D. esculentum* bearings mature spores were randomly collected from both locations. These mature spores are found on the abaxial side of the sporophyll and are indicated by the black or brown-colored sporangia clustered in sori. The fronds were removed using pruning shears and subsequently placed inside a plastic bag.

Drying of the Fertile Pinna

Pinnae that contain mature spores were then stored inside a paper envelope for four to five

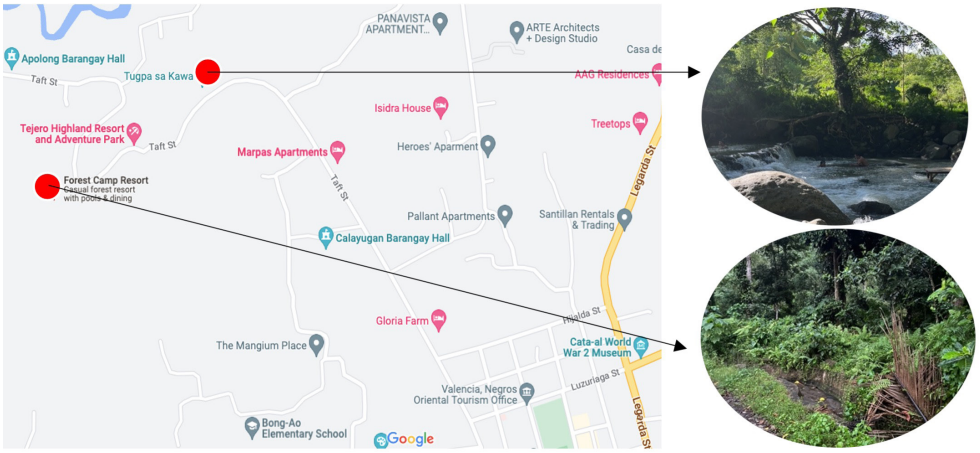

Figure 2. Exact locations of the Kawa River and Forest Camp in Valencia, Negros Oriental.

days. This drying process was needed for the sporangia to release its spores naturally. Once the drying process was complete, spores were then collected aseptically.

Sterilization Process of Potting Soil

The sterilization process was done with the use of an autoclave, also known as a steam sterilizer, to kill any pathogens that can affect the accuracy of the results in the development of *D. esculentum* spores (Darbar, 2007). All substrates and media used for the experiment were autoclaved for one hour.

Preparation of the Potting Media

Five soil media with nine replicates each, including the control group, were used for the experiment (Fig. 3). A plastic container with a lid was used, and the soil substrates were purchased from a local botanical garden.

Media A was composed of potting soil mixed with carbonized rice hull, with a layer of perlite at the bottom. Media B was composed of a layer of perlite at the bottom, and the next layer was composed of potting soil mixed with shredded, sterilized, dried tree fern trunks. Media C, a layer of perlite at the bottom, and the potting soil were mixed with sterilized coco peat. Media D was composed of a layer of perlite at the bottom, and the potting soil was mixed with sterilized peat moss. The control group media were composed of regular potting soil only and a layer of perlite at the bottom. The purchased regular potting soil was composed of regular soil, rice husk, compost, and small sand.

Distribution of *D. esculentum* Spores into the Potting Media

An analytical balance was used to measure the equal distribution of spores in the potting

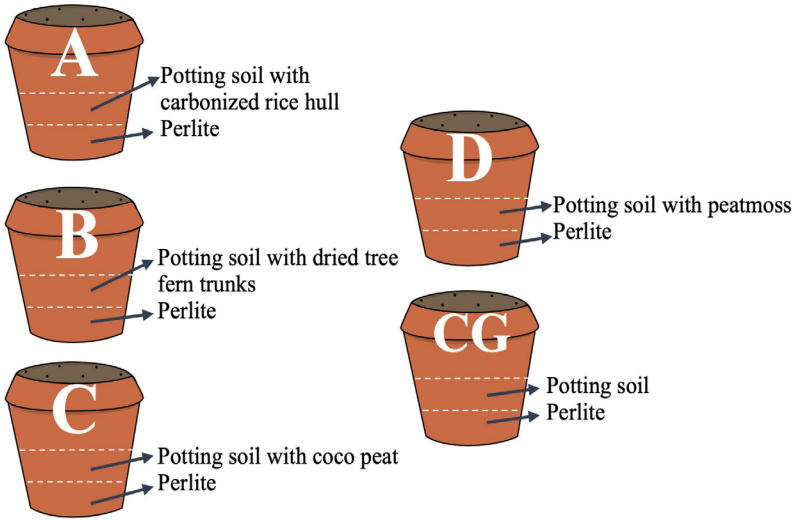

Figure 3. Representation of the Compositions of Each Potting Media

media. An exact amount of 2 grams of naturally released spores for each replicate were measured and distributed evenly into the potting media in a spiral manner. This spiral distribution of spores ensures their maximum exposure to the factors within the media.

Arrangement and Location for the Potting Media

The set-up of the experiment was placed in the Biology Department Plant House at Silliman University, Dumaguete City (Fig. 4). RCBD or the randomized complete block design was followed for the arrangement of the potting medium (Table 1). The set-up was placed in a shady area at the plant house to avoid exposure to direct sunlight.

Table 1. RCBD or Randomized Complete Block Design Arrangement of the Potting Media (CG = Control Group). This was used for the set-up for equal distribution of light.

RANDOMIZED COMPLETE BLOCK DESIGN											
AQUARIUM/TERRARIUM					A	B	C	D	CG	A	B	C
					D	CG	A	B	C	D	CG	A
					B	C	D	CG	A	B	C	D
					CG	A	B	C	D	CG	A	B
A	B	C	D	CG	C	D	CG	A	B	C	D	CG

Measuring the Abundance in the Potting Media

For potting media where the *D. esculentum* spores are already in their gametophyte stage, the abundance was measured with the use of ImageJ software. ImageJ software has been the most commonly used tool for scientific image analysis (Schneider *et al*., 2012). In

Figure 4. Experimental Set-Up at the Biology Department Plant House in Silliman University Dumaguete City.

measuring the area where the gametophyte growth can be seen, the software needed to have a standard measure for the corresponding pixels of the image. The circumference of the potting media is 63.617 cm^2 and was used as the standard measure. All potting media have the same area.

Determination of the pH of the Potting Media

Soil pH is a measure of soil acidity or alkalinity. It is a scale of 0 to 14, with 7 being considered neutral. Values less than 7 indicate acidic soil, whereas values more than 7 suggest alkaline or basic soil. Soil pH is an important component that can affect plant development and nutrient availability in the soil. The mean initial and final pH of the five different potting media was recorded with the use of a 3-in-1 soil tester. The 3-in-1 soil tester can test the soil's moisture, sunlight, and soil pH.

Statistical Data Using One-Way ANOVA Test

One-way ANOVA test was used to determine if there was a significant difference between the gametophyte growth in the potting media. The data used were the abundance values measured in each replicate. Results are shown in Table 2.

Table 2. A Summary of the ANOVA Test Formulas showing the Source of Variation, Sum of Squares, Degrees of Freedom, Mean of Square, and F-Ratio.

ANOVA TEST FORMULAS

Sources of Variation	Sum of Squares (SS)	Degrees of Freedom (DF)	Mean of Square (MS)	F - Ratio
Between treatment (among)	$\Sigma \frac{(\Sigma X)^2}{n} - \frac{(\Sigma\Sigma X)^2}{N}$	K – 1	Between treatment SS/DF	MS Between / MS Within
Within treatment (between)	Total SS – Between SS	N-K	Within treatment SS/DF	
Total	$\Sigma\Sigma X^2 - \frac{(\Sigma\Sigma X)^2}{N}$	N-1		

n = no. of samples in a variable K = no. of variables
N = total no. of samples (all variables

RESULTS AND DISCUSSION

Description of Gametophyte Growth

The gametophyte stage of *D. esculentum* was described as moss-like structures based on qualitative observations. The gametophyte growth shows a colony-like manner due to a small circular or semi-circle formation. The prothallus is the gametophyte stage of ferns. Figure 5 shows the microscopic images of *D. esculentum* prothallus showing the following characteristics: heart-shaped structure and the hairs growing underneath that resemble roots are called rhizoids.

Gametophyte Growth in Different Potting Media

There were five (5) different potting media, with nine (9) replicates each utilized for the experiment. The control group consists of a regular potting mix with carbonized rice hull and has evident gametophyte growth in all replicates. In potting medium A, which consists of mixed regular potting soil and carbonized rice hull, five (5) replicates have gametophyte growth. Potting medium D consists of regular potting mix and peat moss; four (4) out of nine replicates have gametophyte growth.

Soil pH Values

Soil pH was an important variable, especially for fern growth, due to its combined effect on chemical and biological processes (Anh *et al*., 2011). Very little information has been documented on soil propagation of *D. esculentum* spores. Based on Fujita *et al*. (1955), *D.*

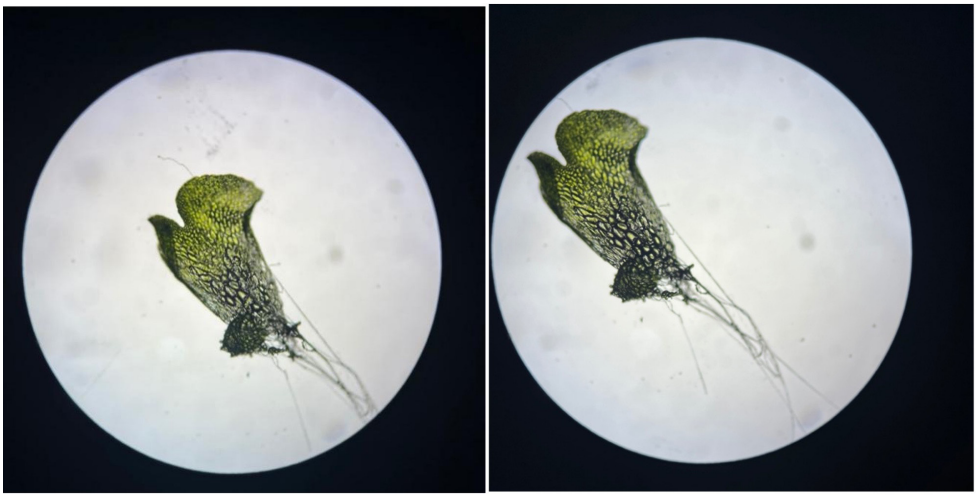

Figure 5. Prothallus of *Diplazium esculentum*.

esculentum thrives on acidic soils or values between 4–7. In in vitro culture, the optimal pH of the medium is 5.8 (Nair *et al*.,2013). Moreover, the spores of leptosporangiate ferns, including *D. esculentum* best germinate in a slightly acidic or neutral pH (Fernandez and Revilla, 2003). The values of potting media A, D, and the control group that exhibited growth are congruent to the previous studies (Fernandez and Revilla, 2003; Nair *et al*.,2003), having a pH range of 5–6. Potting media B and C, having a soil pH of 4–5, did not exhibit growth.

Duration Before Gametophyte Growth

Several factors could affect the spore germination of ferns. Studies show that most species of ferns have a span of 2–3 weeks before gametophyte growth can be observed (Lloyd and Klekowski, 1970; Sheffield *et al*., 2001). Estimation of the general weather condition was conducted during the span of the experiment and was observed that the low-temperature surroundings, due to the consecutive rainy days, have contributed positively to the development of the spores.

The growth rate of the potting media can be correlated to its abundance of gametophyte growth. The control group has the fastest rate of spore germination compared to other potting media, with a span of only 22 days. Potting media A were the next one that exhibited gametophyte growth with a span of 24 days, which is congruent with the results of the study by Medina and Cabras (2015). Potting media D were the last and have the slowest growth rate compared to other potting media, with a span of 45 days, the same as the approximate time rate of the results in the study of Lopez *et al*. (2021).

Late growth of gametophytes was observed on the potting media containing peat moss compared to the rate of growth in the control group and potting media A. This must have been because rice husk is a more effective contributor to the growth of *D. esculentum* spores

compared to peat moss. Studies concerning seed and spore germination with the use of potting media composed of soil with rice husk have shown significant germination percentage (Huang *et al*., 2000; Kumar, 2013; Rollon *et al*., 2017; Taskirawati, 2020) exhibiting rice husk is more efficient in removing toxins and pathogens that could affect the growth of gametophytes. Potting media B and C have not exhibited any spore germination. Studies showed that coco peat is composed of low nutrient content, requiring certain biofertilizers, other substrates, and organic materials to provide more nutrients (Awang *et al*., 2009; Hindersah *et al*., 2022). Media containing dried tree fern trunks were affected because of mold accumulation, destroying primary plant tissues (Romanazzi *et al*., 2016; Sun *et al*., 2019). Table 3 summarizes the duration before the gametophyte growth of each potting medium.

Growth Percentage of Each Potting Medium

Each potting media has a total area of 63.617 cm^2. With the use of ImageJ software, the area occupied by the gametophyte growth was measured. The measured area occupied by the gametophytes corresponds to the abundance of growth in each potting media. With this,

Table 3. Summary of the Obtained soil pH Values, Growth Rate, and the Area Occupied by the Gametophyte Growth (cm2). The number of samples (N), minimum value (MIN), maximum value (MAX), mean of the values, and standard deviation (Std. Dev.).

POTTING MEDIA	PARAMETER	N	MIN	MAX	Mean	Std. Dev.
A	SOIL PH	9	5.000	6.000	5.556	0.527
	GROWTH RATE (days)	9	24.000	27.000	24.600	1.342
	AREA OCCUPIED BY GAMETOPHYTE (cm2)	9	0.000	23.751	4.338	7.734
D	SOIL PH	9	5.000	6.000	5.333	0.500
	GROWTH RATE (days)	9	45.000	52.000	49.250	3.403
	AREA OCCUPIED BY GAMETOPHYTE (cm2)	9	0.000	7.695	1.104	2.556
CONTROL	SOIL PH	9	5.000	6.000	5.444	0.527
	GROWTH RATE (days)	9	22.000	31.000	27.111	4.045
	AREA OCCUPIED BY GAMETOPHYTE (cm2)	9	0.913	28.208	8.634	8.316

it indicates that the bigger the area, the higher the percentage of the abundance of growth.

Figure 6 shows a graphical representation of the growth percentage of each potting media. 12.21% growth percentage of *D. esculentum* gametophytes on potting media A that contains regular potting soil mixed with carbonized rice hull or also known as rice husk ash.

Studies have been conducted on how rice husk ash was used for soil stabilization (Basha *et al*., 2005; Alhassan, 2008; Sudarso and Utomo, 2010; Fattah *et al*., 2013). The study con-

ducted by Sudarso and Utomo (2010) elaborated on how rice husk can improve acid sulfate soils; this kind of soil is not safe for plants. There were improvements in the physical and chemical compositions of acid sulfate soils after they included portions of rice husk ash,

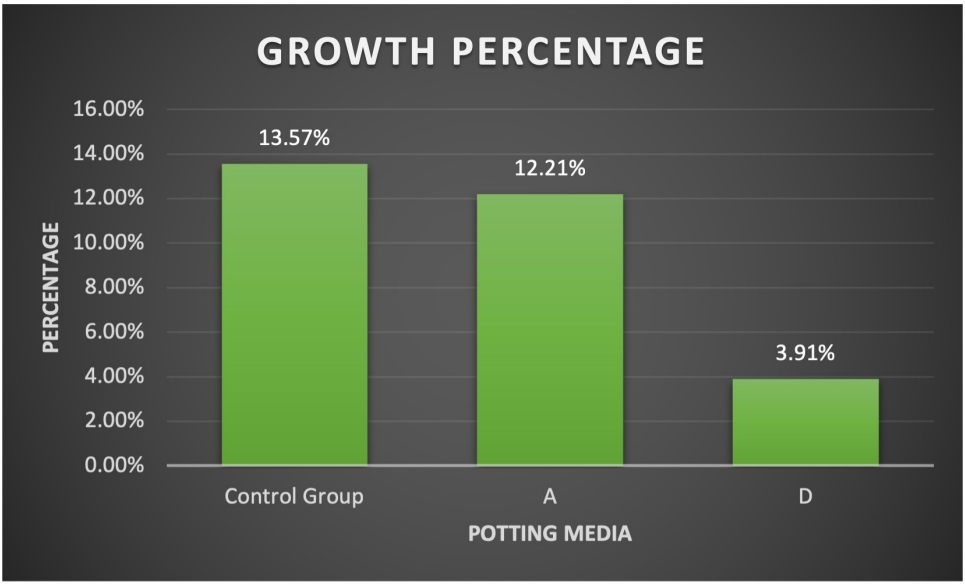

Figure 6. Bar graph representation of the growth percentage of *D. esculentum* spores in each potting media.

rice straw, and rice husk. Rice husk, therefore, is a great contributor to the soil's properties, especially when it comes to the removal of toxins. Praptosuwiryo *et al*. (2015) have used carbonized rice hull in various studies, especially in assessing spore morphology and development of various fern species (*Platycerium wandae*, *Cyathea junghuhniana*, and *Cibotium barometz*) because of the stabilization potential that carbonized rice hull can provide to the soil.

Only 3.9% growth was observed on the potting media D replicates that were composed of regular potting soil and peat moss. Indicating that potting media D has the smallest growth percentage compared to other potting media. A study was conducted by Ponton *et al*. (1990) with the use of in vitro technique during micropropagation of Boston ferns and then later transferred into pots containing different kinds of substrates, one containing peat moss. There was a significant growth of roots on brown peat moss and fast vesicular-arbuscular mycorrhizal (VAM) colonization with black peat moss, therefore concluded that brown peat moss is suitable for fern growth and fungal activity. Peat moss can also be a great substrate for sporophyte growth (Chang *et al*., 2007; Jang *et al*., 2019).

The control group has a growth percentage value of 13.57%. This indicates that regular potting soil is also efficient in the spore germination of *D. esculentum*. Studies show that regular garden soil can ensure a high percentage of spore germination in various species of

ferns (Life, 1907 and Amoroso, 2003). The regular potting soil is composed of rice husk, regular soil, compost, and a small portion of sand. The complex composition of the regular potting mix provided more advantages for the growth of spores compared to other substrates used in the study. Compost is a mixture of organic materials to improve the soil's chemical, physical, and biological properties. Rice husk has been widely used in composting. The results showed abundant growth and a fast rate of spore germination.

No growth was observed on potting media B (dried tree fern trunks) and potting media C (coco peat). Widyastuti *et al*. (2020) explained cocopeat media's disadvantages. Coco peat contains a tiny pore that can prevent water from moving, resulting in greater water availability, which causes difficulties in gas exchange in the media. The small pores of the coco peat are filled with water instead of air, restraining plant growth. Furthermore, contrary to the study of Cabras and Medina (2015), the dried tree fern trunks showed no gametophyte growth, and mold formations were observed. Mold accumulation in plants can affect their growth. Studies showed that a common mold disease caused by *Botrytis cinerea* Pers. ex Fr., a soil-borne fungal pathogen, contaminates crop plantations due to its capability to infect plant tissues through surface injuries inflicted by subsequent handling (Romanazzi *et al*., 2016; Sun *et al*., 2019).

Statistical Data Using One-Way ANOVA Test

A one-way ANOVA test was used to determine if there was a significant difference between the gametophyte growth in the potting media. The data used were the abundance values measured in each replicate. Table 4 shows the pre-ANOVA test, table 5 is the log-transformed data for the ANOVA test conducted after the pre-ANOVA test, and table 6 shows the results using the ANOVA test formulas. Therefore, there is a significant difference in the gametophyte growth using different potting media.

CONCLUSION

This research aimed to investigate the development of *D. esculentum* spores using different potting media. Results have shown that only three out of five potting media show gametophyte growth. These potting media were composed of carbonized rice hull, peat moss, and regular potting soil, each with a different spore germination rate. The carbonized rice hull has the highest growth percentage compared to other potting media, with a value of 12.21%, followed by the peat moss media, which has a value of 3.91%. The growth rate also corresponds to the abundance of growth and shows that the faster the growth, the higher the abundance of the gametophytes. The one-way ANOVA test also provided statistical results based on the abundance of growth in each replicate and gave a statistical decision to reject the null hypothesis. Therefore, there is a significant difference in the rate and abundance of *D. esculentum* spore germination using different kinds of potting media, and the most effi-

Table 4. Pre-ANOVA Test Data Table Using the Abundance of Growth

ABUNDANCE OF GROWTH (cm2)			
SUBJECT	**CONTROL**	**A**	**D**
1	28.208	7.492	1.99
2	2.156	1.385	0.023
3	5.434	4.818	7.695
4	4.63	1.592	0.232
5	7.813	23.751	0
6	7.807	0	0
7	6.193	0	0
8	0.913	0	0
9	14.548	0	0
Variances	69.16045778	59.822048	6.53116253
Sum of Variances	135.5136683		
C	0.510357801		
C table value	0.4775		

Table 5. Log-Transformed Data for the ANOVA Test

LOG TRANSFORMED DATA			
ABUNDANCE OF GROWTH			
SUBJECT	**CONTROL**	**A**	**D**
1	1.450	0.875	0.299
2	0.334	0.141	-1.638
3	0.735	0.683	0.886
4	0.666	0.202	-0.635
5	0.893	1.376	0.000
6	0.892	0.000	0.000
7	0.792	0.000	0.000
8	-0.040	0.000	0.000
9	1.163	0.000	0.000
Variances	0.188	0.249	0.479
Sum of Variances	0.916		

Table 6. Results of the ANOVA Test

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	150.3694467	3	50.1231489	23.823205	2.72364E-08	2.901119584
Within Groups	67.32682551	32	2.1039633			
Total	217.6962723	35				

cient substrate to be used for the spore germination of *Diplazium esculentum* spores is the carbonized rice hull**.**

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